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Efficacy of Leptin Supplementation on Nuclear Maturation and Fertilization Rate of Sheep Oocytes *In Vitro*

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ABSTRACT

The objective of this study was to determine the efficacy of leptin supplementation into maturation medium on nuclear maturation and fertilization rate of sheep oocytes. The maturation process was conducted using a tissue culture medium (TCM) 199 with four supplementation treatments of leptin namely 0 (control), 10, 50, and 100 ng/mL. Fertilization was conducted in the oocytes supplemented with 10 ng/mL and control using 5×10^6 mL^{-1} spermatozoa. At the end of maturation and fertilization processes, the oocytes were stained with 2% aceto orcein to determine nuclear maturation rate and pronuclear development. The results showed that the percentage of oocytes reaching metaphase II (MII) stage significantly increased in the oocytes supplemented with leptin at a dose of 10 ng/mL ($P<0.05$) compared to those supplemented at doses of 50, 100, and 0 ng/mL (93.7±5.9% vs 78.8±4.4%; 72.0±2.6%; 82.1±9.9%). However, fertilization rate of the oocytes supplemented with leptin at a dose of 10 ng/mL and control were similar (72.1±5.5% vs 79.2±7.0%). The data indicated that leptin could improve maturation rate in lower concentration. However, the improved maturation rate of oocytes with leptin supplementation at a dose of 10 ng/mL could not improve the fertilization rate of the oocytes. In conclusion, the supplementation of leptin at a dose of 10 ng/mL could increase the number of oocytes that reached MII stage, but could not increase the fertilization rate.

Keywords: fertilization; leptin; maturation; oocytes; sheep

INTRODUCTION

The success of *in vitro* embryo production is determined by the qualities of the oocytes and the ability of the oocytes to develop into normal embryos. It is therefore that selection of oocytes plays an important role for the success of *in vitro* embryo production. Furthermore, the development of oocytes maturation was found to be influenced by the primary reproductive hormones and the other hormones produced by certain tissues. One hormone that plays a role in supporting the development of oocyte maturation competence is leptin (Cavalcante *et al.* 2013).

Leptin is a hormone secreted by the adipose tissue encoded by the obese gene (Arias-alvares *et al.*, 2010; Sheykhani *et al.* 2016) which has a function in the reproductive organs in the regulation of the ovaries, maturation of the oocytes, and embryonic development (Santos *et al.*, 2012). Leptin receptors are present in the granulose and cumulus cells (Cordova *et al.* 2011; Batista *et al.* 2013; Hu *et al.* 2014; Ding *et al.*, 2017). It was reported that the inter-receptor bonds resulted in improving nuclear maturation and oocyte fertilization up to the stage of embryonic development (Jia *et al.*, 2012). Furthemore, Craig *et al.* (2004) explained that leptin played a role in the process of oocyte maturation in vitro by activating the phosphodiesterase of 3B (PDE3B) enzyme. This enzyme can decrease cyclic adenosine monophosphate (cAMP) (Zhao *et al.*, 1998), thus inducing germinal vesicle breakdown (GVBD) that affects the oocyte maturation. In addition, leptin also affects the maturation promoting factor (MPF) through the activation of mitogen-activated protein kinase (MAPK) (Kakisina & Indra, 2008). It is well known that MAPK plays a role in activating MPF through the production of cyclin B (Craig *et al.*, 2004) and $p34^{cdc2}$ (Villanueva & Myers, 2008). The activated MPF will further stimulate a series of cellular reactions to achieve GVBD, chromosomal condensation, spindle formation, and cytoplasmic maturation (Hunter 2000). The process sequence is necessary to support the nuclear maturation from the germinal vesicle stage (GV) to metaphase II (MII) (Ohashi *et al.*, 2003), resulting in a competent oocyte.

Several studies on the effectiveness of leptin on oocyte maturation to improve the development of the embryos in bovine (Jia *et al.*, 2012), horse (Congsiglio *et al.*, 2009), buffalo (Panda *et al.* 2017), and pig (Moreira *et al.* 2013) were reported. However, the effect of leptin on the oocytes maturations in sheep has not been reported. Therefore, this study was conducted to determine the effectiveness of leptin supplementation in maturation

medium on nuclear maturation and fertilization rate in sheep.

MATERIALS AND METHODS

Oocytes Collection and Maturation *In Vitro*

The sheep ovaries were obtained from the local slaughterhouse and transported to the laboratory in 0.9% NaCl (S9888, Sigma-Aldrich, St. Louis, USA) supplemented with 0.1 mg/mL Streptomycin (S9137, Sigma-Aldrich, St. Louis, USA) and 100 IU/mL penicillin (P4687, Sigma-Aldirch, St. Louis, USA) (Hasbi *et al.* 2017) at 35-37°C (Karja *et al.,* 2013). In the laboratory, the ovaries were washed twice using 0.9% NaCl. The oocytes were collected by slicing technique in phosphate buffered saline (PBS) supplemented with 0.3% bovine serum albumin (BSA) (A7030, Sigma-Aldrich. St. Louis, USA), 100 IU/mL penicillin, and 0.1 mg/mL streptomycin. Only oocytes with compact cumulus cells and having more than three layers of cumulus cells and homogenous cytoplasm were used in this study. Collected oocytes were washed three times and matured in vitro in tissue culture medium (TCM-199) (M4530, Sigma-Aldrich, St. Louis, USA) supplemented with 0.3% BSA, 10 IU/mL pregnant mare serum gonadotrophin (PMSG) (Kyoritsu Seiyaku, Japan), 10 IU/mL human chorionic gonadotrophin (hCG) (Intervet Boxmeer-Holland), and 50 μg mL gentamicin (G1264, Sigma-Aldrich, St. Louis, USA) (Setiadi & Karja, 2013). Maturation medium was supplemented with leptin (L-4146, Sigma-Aldrich,St. Louis, USA) at doses of 10, 50, and 100 ng/mL and control without leptin supplementation. The oocytes were matured in sterile petridish 35×10 mm (Nunclon, Denmark) in the form of a drop of 100 μL (10-15 oocytes) which were covered by mineral oil (M5310, Sigma-Aldrich,St. Louis, USA). The oocytes were incubated in 5% CO_2 at 38.5ºC in humidified air for 24 hours.

Maturation Rate

The cumulus cells were removed from the oocytes by using 0.25% hyaluronidase (H3506, Sigma-Aldirch, St. Louis, USA) in a sterile petridish in the form of a drop of 100 μL at room temperature and repeated pipetting with an appropriate pipette adjusting to the size of the oocytes. The denuded oocytes were washed in 0.3% PBS+BSA and placed on an object glass which was overlaid with 0.3% PBS+BSA then covered by a cover slip. Then the slide was fixed into a solution mixture of absolute ethanol (K44151883 303, Merck, Germany) and acetic acid (K45626263 420, Merck, Germany) in the ratio of 3:1 for 48-72 hours. The slide was then stained with 2% aceto-orcein (O7380, Sigma-Aldirch, St. Louis, USA) for five minutes and washed in 2% acetic acid. Nuclear maturation of the oocytes were examined under a phase contrast microscope (Olympus IX 70, Japan).

In Vitro Fertilization of Oocytes

The matured cumulus oocyte complexes (COCs) produced by supplementation of leptin at doses of 0 (control) and 10 ng/mL were fertilized using the frozen semen. The frozen semen in straw (IMV, France) was thawed in a waterbath with temperature of 37°C for 30 seconds. For selection and capacitation, the semen was diluted with fertilization medium and washed by centrifugation (Suzuki *et al.,* 2000) at 1800 G for 5 minutes. The supernatant was removed and the pellet was diluted using fertilization medium to the final 5×10^6 mL^{-1} concentration of spermatozoa (Adelia *et al.,* 2017). The matured COCs were washed twice with fertilization medium and then incubated with the spermatozoa in 5% CO_2 at 38.5°C in humidified air for 14 hours. Fertilization rate was evaluated with 2% aceto-orcein as was described above to determine the formation of pronucleus (PN).

Statistical Analysis

The percentage of nuclear maturation were analyzed using ANOVA. If there was any significant different, further testing was continued by using Duncan's Multiple Range Test (DMRT). The fertilization rate was analyzed by independent sample t-test. Difference at probability of P≤0.05 was determined as a significant difference.

RESULTS

Nuclear Maturation Rate

The chronologies of nuclear maturation are shown in Figure 1 i.e., germinal vesicle stage (GV), germinal vecicle breakdown (GVBD), metaphase I (MI), and metaphase II (MII). The percentages of oocytes reaching the MII stage in maturation medium supplemented with leptin at doses of 0, 10, 50, and 100 ng/mL were 82.1±9.9%, 93.7±5.9%, 78.8±4.4%, and 72.0±2.6%, respectively (Table 1). The results of the experiment showed that leptin supplementation at a dose of 10 ng/mL was able to improve the nuclear maturation rate to reach MII stage that was significantly higher (P<0.05) compared to the other doses and control.

Fertilization Rate

The success of the fertilization in vitro is characterized by the formation of two or more pronuclei (Figure 2). The normal fertilization was categorized when the oocyte had two pronuclei. If the oocyte had more than two pronuclei it was catagorized as a polyspermy. The percentage of fertilized oocytes to form two pronuclei or more than two pronuclei on a maturation medium supplemented with leptin at a dose of 10 ng/mL (79.2±7.0%) was numerically higher than that in the control (72.1±5.5%) even though it was not statistically significant. In additon, the normal fertilization rate (to form two pronuclei) was in line with the total fertilization rate i.e., numerically higher in medium supplemented with leptin at a dose of 10 ng/mL (71.9±7.9%) compared to control without leptin supplementation (62.9±4.7%). In addition, the polyspermy rate was lowered in the medium supplemented with leptin at a dose of 10 ng/mL

(7.3±7.7%) compared to those without leptin supplementation or control (9.2±6.5%) (Table 2).

DISCUSSION

Nuclear Maturation Rate

The results found in the present study are in line with the results of the studies in pig (Craig *et al.*, 2004), bovine (Arias-alvares *et al.*, 2010; Jia *et al.*, 2012), buffalo (Sheykhani *et al.* 2016), and rabbit (Khaki *et al.*, 2013) reporting that the concentration of 10 ng/mL of leptin supplementation in the maturation media is the optimum dose to increase the nuclear maturation rate to reach MII stage charaterized by the formation of polar body I. Furthermore, these data are in line with the results reported by Kamalamma *et al.* (2015) who used leptin to culture preantral sheep follicles to improve oocyte maturation. Improvement of nuclear maturation by leptin is occurred through the receptors present in

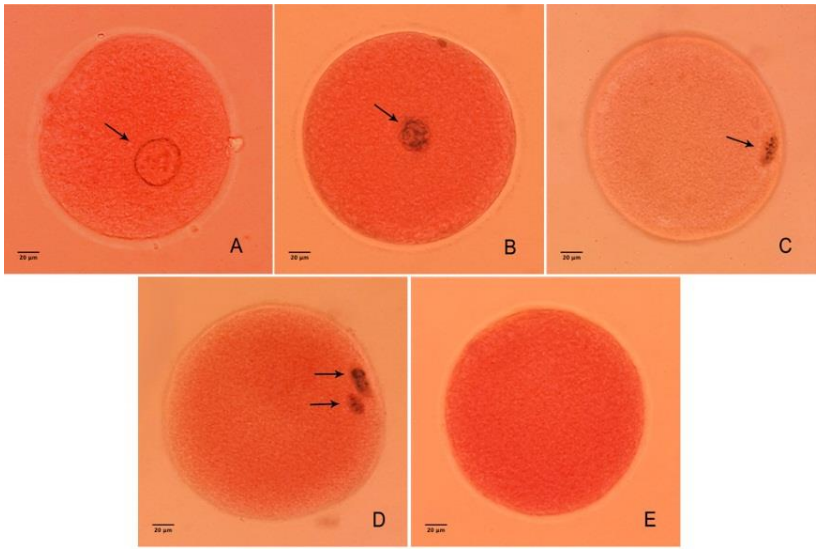

Figure 1. Development of nuclear maturation rate of oocytes in various concentrations of leptin supplementation in the maturation medium. A= germinal vesicle, B= germinal vesicle breakdown, C= metaphase I, D= metaphase II, E= degeneration (arrows); The image was taken by using a phase contrast microscope with 200x magnification.

Table 1. Nuclear maturation rate of sheep's oocytes in maturation medium supplemented with leptin at various doses

Treaments	No. of oocytes	Nuclear maturation rate (% average ±SD)				
		GV	GVBD	MI	MII	Deg
Control	87	1(0.6±1.7)	7(6.5±7.7)^{ab}	9(11.5±11.2)^{ab}	71(82.1±9.9)^{b}	0(0±0)
P1	85	0(0±0)	3(2.5±4.5)^{b}	4(3.8±4.9)^{b}	78(93.7±5.9)^{a}	0(0±0)
P2	86	0(0±0)	5(5.6±5.4)^{ab}	13(15.6±4.9)^{a}	68(78.8±4.4)^{bc}	0(0±0)
P3	84	2(1.8±3.3)	9(10.5±5.8)^{a}	11(14.4±6.9)^{a}	61(72.0±2.6)^{c}	1(1.3±3.4)

Note: Means in the same columns with different superscripts differ significantly (P<0.05). Control=leptin supplementation at a dose of 0 ng/mL, P1= leptin supplementation at a doses of 10 ng/mL, P2= leptin supplementation at a dose of 50 ng/mL, P3= leptin supplementation at a dose of 100 ng/mL. GV= germinal vesicle, GVBD= germinal vesicle breakdown, MI= metaphase I, MII= metaphase II, DEG= degeneration.

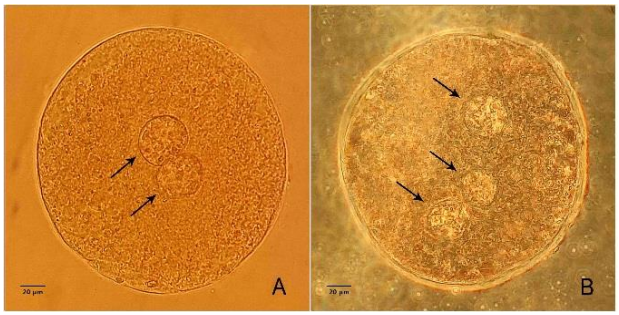

Figure 2. Pronuclear development of sheep oocyte fertilization supplemented with leptin. A= 2 pronuclei, B= >2 pronuclei; The image was taken by using a phase contrast microscope with 200x magnification.

Table 2. Fertilization rate of sheep's oocytes supplemented with leptin at doses of 0 (control) and 10 ng/mL

Treatments	No. of oocytes	Pronuclear Formation		Fertilization rate (%)
		2 PN (%)	>2PN (%)	
Control	107	68(62.9±4.7)	10(9.2±6.5)	78(72.1±5.5)
Leptin 10 ng/mL	106	76(71.9±7.9)	8(7.3±7.7)	84(79.2±7.0)

Note: 2PN= two pronuclei, >2PN= polyspermy (more than two pronuclei), total fertilization: the number of oocytes that could form 2PN or > 2PN of the total number of fertilized oocytes.

the cumulus cells (Ye *et al.,* 2009). In addition, Craig *et al.* (2004) suggest that the mechanism of leptin in supporting nuclear maturation is carried out through the increased MAPK pathways.

Furthermore, Arias-alvales *et al*. (2010) suggest that leptin mechanism in improving nuclear maturation is done through its receptors effects on the activation of the Janus Kinases pathway (JAK), signal transducers and activators of transcription (STAT) (Merhi *et al.* 2013), and mitogen-activated protein kinase (MAPK). MAPK activates MPF by stimulating the synthesis and translation of cyclin B then binds to $p34^{cdc2}$ (Kakisina & Indra, 2018), thus stimulating meiotic reinitiation to achieve cytoplasmic maturation (Hunter 2000), and supports nuclear maturation from GV to MII stage (Ohashi *et al.,* 2003).

The results of this study showed that supplementation of leptin at doses higher than 10 ng/mL (50 and 100 ng/mL), could not improve nuclear maturation rate compared to the control group. Nevertheless, some studies were unable to explain the mechanisms of higher doses of leptin supplementation (50 and 100 ng/mL) in improving the nuclear maturation rate of the oocytes. Kamalamma *et al.*(2015) and Yorio *et al.* (2013) state that leptin supplementation at a low dose (10 ng/mL) can activate STAT3 and ERK1 (MAPK) while at higher doses, 300-500 ng/mL, only STAT3 was activated. The results of this study are in line with some previous studies that leptin supplementation at a dose of 100 ng/mL does not increase the maturation rate of bovine oocytes (Jia *et al,.* 2012).

Fertilization Rate

Data on the fertilization rate in this study showed that the supplementation of the maturatiojn medium with leptin at a dose of 10 ng/mL could improve nuclear maturation significantly (93.7%), although it did not significantly improve fertilization rate compared to the control group (79.2% vs 72.1%). Hyttel *et al.* (1997) suggested that fertilization success would not be optimal if the cytoplasmic maturation, the chromosomal condensation, and the spindle formation were incompetent. Furthermore, Jin *et al.* (2009) reported that leptin did not affect the formation of microfilaments and actin filaments that were essential for pronuclear formation. In contrary, Jin *et al.* (2009) revealed that supplementation of leptin into the maturation medium could accrelarate the increase in MAPK, thus increasing the pronuclear formation. Nevertheless, the results of Setiadi *et al.* (2009) showed that although MPF and MAP kinase concentrations were high in pig oocytes, these high concentrations did not correlate well with the fertilization rate capability.

CONCLUSION

Low dose of leptin (10 ng/mL) could improve the number of oocytes reaching the MII stage. However this low dose of leptin supplementation in the maturation medium could not improve fertilization rate.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in this study.

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